AD_____

AWARD NUMBER: W81XWH-04-1-0029

TITLE: Identification of New EGR1 Target Genes That Regulate Radiation Responses in Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: Shilpi Arora, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

REPORT DATE: July 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2006	Annual Summary	1 Jul 2004 – 30 Jun 2006

4. TITLE AND SUBTITLE

Identification of New EGR1 Target Genes That Regulate Radiation Responses in Prostate Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0029

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Shilpi Arora, Ph.D.

E-Mail: angiem@burnham.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Burnham Institute
La Jolla, California 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
I have studied the induction of early stress response gene, Egrl under various stress conditions in prostate cancer cells. I chose the induction of these cells by **UV** treatment for identifying target genes of Egrl under this stress condition. I used ChIP on Chip (using promoter arrays) to identify these target genes. I helped in preparation of these promoter arrays which have -12,000 promoter sequences on them and then went ahead to standardize all the protocols to achieve minimum false positive results. These experiments helped me to identify various known and unknown targets of Egrl such as **IGF1,** MAP2K1, **CDKNIB, CCNB2,** ADAR and **PAX3.** We identified 177 target genes after the statistical analysis and verified that at least 68% of them contained Egrl binding consensus sites in their promoters. Ten of these genes were validated by conventional Chip and by Q-RT-PCR analysis. The major pathways involved were Tissue Development/ Cellular Movement/Gene Expression, Cell Death, cell Cycle, Cell signaling, Cellular Development, Cancer/Cell-To-Cell Signaling and Interaction. The results show that Egrl induction upon **UV** irradiation leads to a cascade of events in prostate cancer cells. Further studies on these target genes are underway to study the effect of these
genes after irradiation of cells.

15. SUBJECT TERMS
Eqr1, Chip, promoter arrays, UV stimulus

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

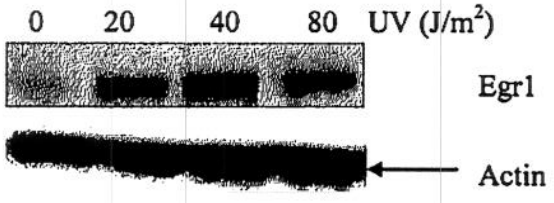
INTRODUCTION

The Zinc finger transcription factor Egr-1 [1, 2] (also called Zif268, NGFIA, Tis8 and Krox24) was discovered independently by several laboratories (including our laboratory) in searching for genes essential for growth, proliferation and differentiation. It can be elicited in response to a diverse variety of extra cellular signals, including growth factors, cytokines, phorbol esters, irradiation of all wavelengths, chemotherapy drugs, and stresses of many kinds. Egr1 is the most responsive (mRNA induced within 5' and protein level peaking at 60 min) of the stress activated genes. It binds to the consensus DNA sequence: GCGG/TGGGCG and many variants of this, most recently GCGGCGGCG. WT1, the Wilm's tumor suppressor gene, can bind to the same sequences as Egr1 as well as to quite unique ones. The competition between WT1 and Egr1 is likely to play a role in prostate tissues where Egr1 and WT1 are co-expressed [3].

In prostate cancers, Egr1 is found to be expressed at high levels [4-6], but is low or absent in normal prostate tissue. Moreover, the level of Egr1 increases with the degree of malignancy as measured by the Gleason score of the tumor [4]. This is unique to prostate cancer because in mammary, lung and glial tumors, Egr1, is not over-expressed. Mouse models using knockout and transgenic mice support the conclusion that Egr1 is required for tumor progression [3]. The role of Egr1 and its known target genes have recently been reviewed [7]. Therefore, we wanted to identify targets of Egr1, upon treating prostate cells with various stress stimuli. For accomplishing this task, ChIP on Chip, (ChIP=chromatin immunoprecipitation), a very useful technology. ChIP works by crosslinking the transcription factor to its target genes followed by immunoprecipitation to capture the bound DNA. The captured DNA fragments are then labeled with a fluorescent dye and hybridized to an array of human gene promoters. Those spots that give strong hybridization signals are deemed Egr1 target genes and then validated by conventional gene expression analysis. Hence, promoter arrays have provided us with a platform to determine the signal transduction pathways that are induced as a result of Egr1 induction in prostate cancer cells by various stress stimuli.

RESULTS (in reference to the Statement of work)

Task 1. To test prostate cancer cell lines with nonfunctional p53 for their biological responses to ionizing radiation (IR)(Months 0-4). A, B. DU145 and several prostate cancer cells that I tested, responded only slightly to **ionizing** radiation to give a very low induction of Egr1. So, I focused on induction of Egr1 by **UV** irradiation treatment in DU145 cells. M12 prostate cancer cells also showed maximal induction of Egr1 protein 2 hours after UV radiation (40 J/m^2). We showed in last years report that Doxorubicin, the chemotherapy agent also induces Egr1 in a similar time course.



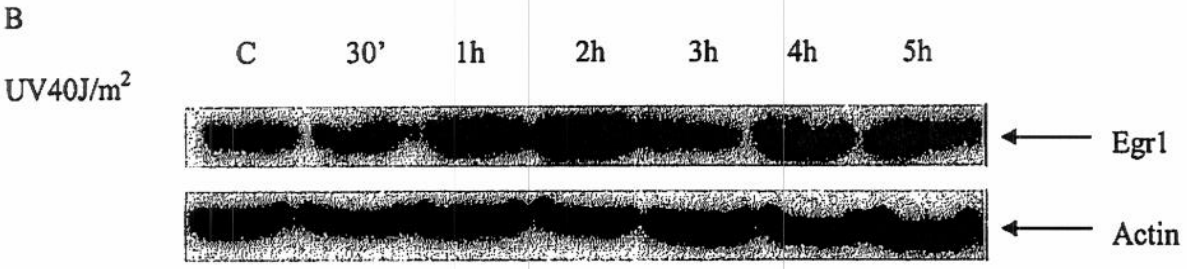


Figure 1: Egr1 induction on UV treatment in DU145 cells. A. Dose Course of UV treatment, cells were collected 2 h after induction with different doses of UV (Control, 20J/m^2, 40J/m^2 and 80 J/m^2 respectively), B. Time course of UV treatment, Cells were treated with 40 J/m^2 and collected after varying time points (Control, 30 min, 1h, 2h, 3hr, 4hr and 5 hr respectively).

Task 2. . To determine whether Egr1 plays an important role in such biological responses. (Months 5-8)

We performed this task for the induction of Egr1 on Doxorubicin treatment in $Egr1^{-/-}$ and $Egr1^{+/+}$ MEF cells (year 1 report) showing that the cells containing Egr1 are more sensitive to Doxorubicin treatment and apoptosis.
Meanwhile, a colleague, Dr Jianxiu Yu in the lab showed that when DU145, M12 and other cell types are treated with expression vectors for Egr1 or some of its target genes (p53 and p73) apoptosis occurs to different degrees (J.Yu., V. Baron, D. Mercola, T. Mustelin, and ED Adamson, in press). He also showed that Etoposide (chemotherapy drug) induces Egr1 and interfering RNA to Egr1 prevents apoptosis, showing that Egr1 and its target genes all contribute to apoptosis. His work (supported by a DOD fellowship) showed that Egr1, p73 and p53 induce each other in a feedback mechanism. Therefore, we conclude that Egr1 is an important transcription factor for stress response.

Task 3. (Years 1 and 2) To identify Egr1 target genes upon irradiation or chemotherapy drug treatment, using ChIP on a chip.
I spent most of the end of year 1 and year 2, optimizing the exact procedure for getting reproducible results for ChIP on Chip,. a technique that has become widely used for demonstrating the binding of a transcription factor to the promoter regions of the DNA of the target genes of that transcription factor.) When we started this study, I was using the promoter arrays with ~3000 promoter sequences on them but during the course of this one year we have constructed a newer version of the promoter array with more than 12,000 promoter sequences on them. The primer sets used for PCR were a kind gift from Dr. Michael J. Birrer (Chief Molecular Mechanism Section,

NCI). The new promoter array consists of 2 slides that contain the entire gene set spotted in triplicates. I worked closely with the groups of Dr. Michael McClelland and Dr. Dan Mercola (Sidney Kimmel Cancer Center, La Jolla, currently at University of California, Irvine) to construct these promoter arrays.

I tested various different stimuli to identify new targets of Egr1 using ChIP on Chip under different kinds of stress for example UV, serum starvation and doxorubicin. Herein, I present the results for UV induction and these experiments have worked well and seem to give consistent results in dye swap experiments (Figure 2).
20 ng of Total Input Control (TIC) DNA (from DU145 cells) was taken and then amplified after sequenase reactions using random PCR primers and subsequently labeled with fluorescent dyes, Cy3 or Cy5 respectively. These samples were hybridized with Chromatin immunoprecipitated DNA from UV treated cells (using anti-Egr1 antibody, *sc-110,* labeled with cy3 and cy5)

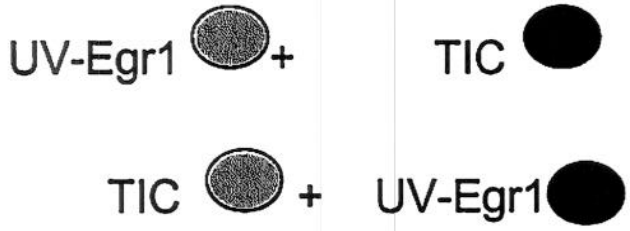


Figure 2: Schematic representation of the hybridized samples

Before proceeding with the hybridization, I checked the chromatin immunoprecipitated sample by performing a Western Blot gel (Figure 3).

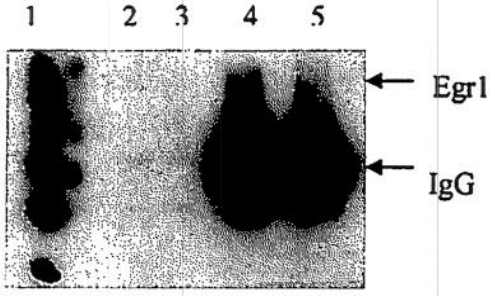


Figure 3: Western Blot analysis of chromatin immunoprecipitated (with Egr1 antibody) DU145 cells. 1. Protein Ladder 2. After IP supernatant from UV treated cells, 3. After IP supernatant from control cells, 4. UV treated sample, 5. Control sample.

After the confirmation, DNA was amplified using random primers (Figure 4).

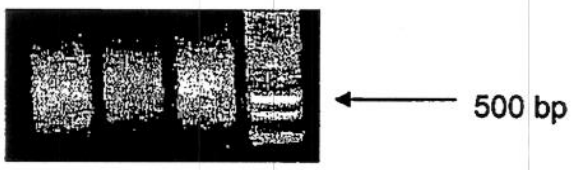
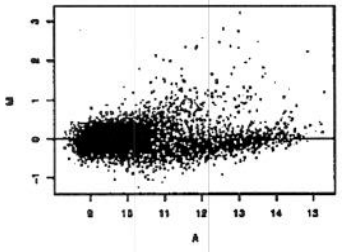


Figure 4. Confirmation of the yield and size of amplified PCR products after ChIP using Egr1 antibody.1, Total Input Control DNA. 2, Control sample, 3. UV treated sample

These samples were then used in the dye swap experiment as explained above and the data was evaluated using the R statistical program. This generated a list of target genes of the Egr1 transcription factor. Each spot represents a gene:-

A



B

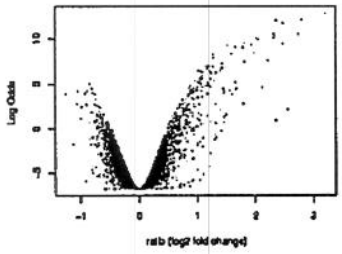


Figure 5. A. MA plot of promoter array hybridization intensities of ChIP products. M = log2 R − log2 G and A = (log2 R + log2 G)/2. B. Significance ("volcano") plots (Jin et al. 2001 and Wolfinger et al. 2001) of the hybridization intensity data for ChIP products of DU145 cells treated with UV radiation.
The genes on the right-hand side of the "volcano?" plot (B) in the upper right extended streak are those that are unique to the irradiated sample.

After statistical analysis 177 differentially expressed genes were selected which had positive B values, positive M values, as well as probability differences of $p<0.001$. All these were then checked to see whether they contained Egr1 binding consensus sites. One hundred twenty of the 177 genes (68%) contained Egr1 binding sites in their promoters. All the 177 genes were checked for pathway networks using Ingenuity pathway analysis software. These genes and the pathways are listed in Table 1 (Appendix 1).

10 genes (containing consensus Egr1 site in the promoters) were randomly selected for further validation (Table 2) using conventional ChIP and Q-RTPCR analysis.

Table 2: Genes validated by Q-RTPCR

Genbank ID	Name	EBS	Description
NM_006044	HDAC6	2	histone deacetylase 6
NM_015840	ADAR	1	adenosine deaminase, RNA-specific isoform ADAR-b
NM_012448	STAT5B	2	LGP1 homolog
NM_006264	PTPN13	2	protein tyrosine phosphatase, non-receptor type 13 isoform 2
NM_006860	RABL4	2	RAB, member of RAS oncogene family-like 4
NM_002744	PRKCZ	2	protein kinase C, zeta
NM_032871	TNFRSF19L	2	tumor necrosis factor receptor superfamily,
NM_005474	HDAC5	2	glucose-6-phosphatase catalytic subunit 3
NM_003559	PIP5K2B	3	proteasome beta 3 subunit
NM_002755	MAP2K1	2	mitogen-activated protein kinase kinase 1

EBS: Egr1 binding site

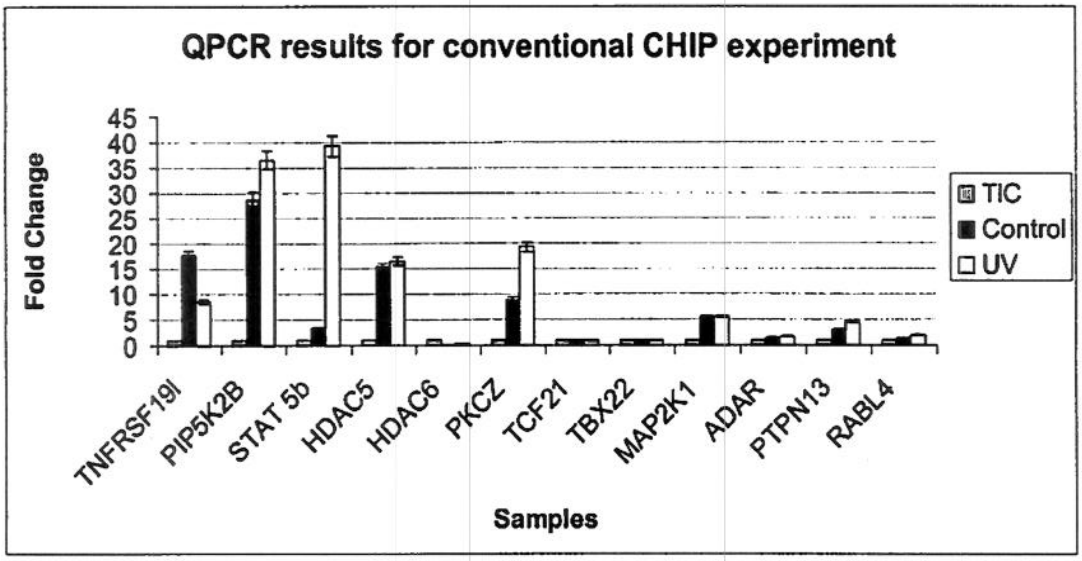


Figure 6: Conventional ChIP was performed for all the 10 genes selected and it was observed that there was definite enrichment of DNA for the all putative Target genes except HDAC 6. TCF21 and TBX22 were controls (these genes do not have any Egr1 binding sites in their promoters, and UV did not change their regulation).

QRT-PCR analysis for mRNA levels of all the 9 genes tested, enrichment had occurred for 2 of the genes that showed up-regulated mRNA (MAP2K1 and TNFSF19L) while 6 showed down-regulation at the transcript level (PIP5K2B, STAT 5B, PKCZ, ADAR, PTPN13 and RABL4).

Key Research Accomplishments.

1. The above data are being repeated, verified and refined for publication as a paper entitled "Identification of Egr1 target genes in prostate cancer cells upon UV irradiation".

2. I am preparing the data for publication with the help of a biostatistician in the department

Reportable Outcomes.

No publications have resulted so far, but training in this difficult technique has been accomplished. Bioinformatics techniques will be acquired and applied to the data and should result in data that will add Egr1 as a major transcription factor that could be used for therapy by understanding the control of tumor growth that the Egr1 gene can accomplish when used under the correct conditions.

Conclusions

The tumor suppressor transcription factor Egr1 (early growth response gene) is a multifaceted gene whose prominent feature when used in tumor therapy is its rapid response to stress stimuli such as radiation and chemotherapy drugs. Under these conditions, Egr1 will be activated and will cause the apoptosis of tumor cells. When used in conjunction with controlling factors, it has already been shown to be useful both for its promoter and for the properties of the protein in therapeutic applications. However, further refinements will be needed to direct the multiple functions of this gene product.

APPENDIX 1.

TABLE 1: Analysis of the major gene Pathways that were initiated by the UV Induction of Egr1in DU145 cells.

REFERENCES

1. Christy, B., Lau, L. F. & Nathans, D. (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "Zinc finger" sequence, *Proc.Natl.Acad.Sci.USA. 85*, 7857-7861.

2. Sukhatme, V. P. (1990) Early transcriptional events in cell growth: the Egr family., *J.Am.Soc.Nephrol. 1*, 859-866.

3. Abdulkadir, S. A., Qu, Z. C., Garabedian, E., Song, S. K., Peters, T. J., Svaren, J., Carbone, J. M., Naughton, C. K., Catalona, W. J., Ackerman, J. J. H., Gordon, J. I., Humphrey, P. A. & Milbrandt, J. (2001) Impaired prostate tumorigenesis in Egr1-deficient mice, *Nat. Med. 7*, 101-107.

4. Eid, M. A., Kumar, M. V., Iczkowski, K. A., Bostwick, D. G. & Tindall, D. J. (1998) Expression of early growth response genes in human prostate cancer, *Canc. Res. 58(11)*, 2461-2468.

5. Mohiuddin, M., Ahmed, M. M., Venkatasubbarao, K., Fruitwala, S., Rangnekar, V., Cross, P. & Weinstein, M. (1997) Egr-1 expression and mutation analysis in prostate tumors. (Abstract), *Proc. Amer. Ass. Canc. Res. 38*, 427.

6. Thigpen, A. E., Cala, K. M., Guileyardo, J. M., Molberg, K. H., McConnell, J. D. & Russell, D. W. (1996) Increased expression of early growth response-1 messenger ribonucleic acid in prostatic adenocarcinoma, *J Urol. 155(3)*, 975-981.

7. Adamson, E. D. & Mercola, D. (2002) Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. [Review] [141 refs], *Tumour Biology. 23*, 93-102.

APPENDIX 1

TABLE 1

ID	genes	focus genes	top functions
1	AKT3, **BMP4**, **BTG1**, **CASP1**, **CD40LG**, **CDKN1B**, **CSN2**, **CXCL6**, **CXCL9**, DUSP2, DYRK1B, **GLI2**, HAPLN1, **HDAC5**, **HK1**, **IFRD1**, **IGF1**, **JAG1**, **MAP2K1**, **MAP3K14**, **MEF2C**, MEOX1, **MKNK1**, **MMP9**, NODAL, **OLFM1**, **PAX3**, PKIA, **PRKCZ**, PSME1, **RPS6KA2**, SFRS3, SP7, **STAT5B**, T	24	Tissue Development, Cellular Movement, Gene Expression
2	ABCC3, **ACVR1C**, **ADAR**, **CD1B**, **COL4A5**, DUSP9, ECH1, **GSTA3**, GSTM2, **IL1R2**, **KLF7**, LEP, LSS, MAPK14, MAPKAPK5, NFAM1, NUTF2, PEMT, **PIP5K2B**, **PRSS23**, **PTPRCAP**, RAN, RPS6KA4, SC4MOL, SC5DL, SEPP1, SERPINA12, **SLC15A1**, SMAD2, SPTA1, **SREBF2**, TNF, TNN, **TRPA1**, ZAP70	13	Cell Death, Connective Tissue Disorders, Lipid Metabolism
3	**ABCB4**, **AKAP6**, **BLK**, BUB1, **CCNB2**, CHRNA1, **CHRNA2**, CTSW, **DEGS1**, DGKA, **GCAT**, GNL3, HCN1, HCN2, **HCN4**, **HDAC6**, ID3, IL2, MAD2L1, MAD2L2, NOL1, PDE4B, POU4F1, PRKAR2A, **PSMB2**, PTEN, RBBP6, RBL2, **REV3L**, **SEMA7A**, SHOX, **STRN3**, TEP1, TP53, TP53RK	13	Cell Cycle, DNA Replication, Recombination, and Repair, Cellular Compromise
4	AEBP1, ALDH3A2, **CTNNA2**, CTNNB1, D13BWG1146E, **DYRK2**, E2F6, **FES**, FHL1, **FRAT1**, **GDF5**, GNAI3, **GNAZ**, GNB5, **GNG7**, GNG11, GNG12, **GRIK1**, HTRA1, KLF16, MAPK1, MAPT, MTNR1A, NFATC2, NPTX1, **NPTXR**, OPRD1, PCYT1A, **PTPRR**, R9AP, **RGS11**, RGS20, SP1, TRD@, **ZNF43**	12	Cell Signaling, Nervous System Development and Function, Nucleic Acid Metabolism
5	**ACTB**, ACTL6B, **AZGP1**, C19ORF10, CD33, CDH1, CMAS, CXCL12, DECR1, ERBB2, F11R, GMNN, H1F0, **HOXB4**, IFNG, **IGSF1**, **IL1R1**, INHBA, MUC4, **PRDM2**, PRDX2, PTPRB, **PVRL1**, PVRL3, **RANBP5**, RB1, **RPL23A**, **RYR3**, SMARCB1, TMSB4X, TPD52, **TPD52L1**, TPO, TXK	11	Cellular Development, Hematological System Development and Function, Cell-To-Cell Signaling and Interaction
6	ADM2, AKT1, ANKRD1, **C1QB**, C1QC, CALCA, **CALCRL**, **CHFR**, CPE, CRYAA, CRYAB, CRYBB2, **CRYGC**, E2F4, G3BP2, HSD11B2, INS1, **MC3R**, NDUFB9, NFKBIA, NFKBIB, **NOS1**, **PCSK1N**, PDE3B, PDE8A, **POLR2H**, POMC, **PTPN13**, RAMP1, RCP9, RPS6KB2, **SRRM2**, UBE1, UMPS, **YBX1**	12	Cancer, Cell-To-Cell Signaling and Interaction, Skeletal and Muscular Disorders
7	ALPP, ASC2, **ATG5**, ATG12, AVPR1A, BDKRB1, **CD164**, **CEACAM8**, **CITED4**, CSF3, CSTA, **CYSLTR1**, **DNAJB9**, EDF1, ERCC1, FADD, FOLR2, FOS, **GCHFR**, GCLM, GSTA1, HAS1, HIF1A, IL1B, JUN, **LAMP2**, MAFF, NFKBIZ, NMB, **OXTR**, **RGL2**, SCIN, SERP1, SLC10A2, XBP1	10	Cardiovascular System Development and Function, Cellular Movement, Gene Expression

8	AGRN, **ALS2CR2**, **AOX1**, APBA2, APBB2, APBB3, **APLP2**, APP, CEBPB, **CSTB**, DCX, **DNAL4**, DYNC1I2, DYNLT1, FSTL1, GLUL, GPI, HERPUD1, HMOX2, HRK, IFITM3, ITM2B, KRAS, **MAP2**, MAPK8, NDE1, NRG1, **PAFAH1B1**, PFKFB1, PRDX4, **PRKACA**, SLC10A1, SPON1, **SPTAN1**, THOP1	9	Cell Death, Nervous System Development and Function, Genetic Disorder
9	**MYOC**	1	Ophthalmic Disease
10	**DPPA4**, SH3GL1	1	Cancer, Hematological Disease, Cell Morphology
11	MAPKAPK2, **OVGP1**	1	DNA Replication, Recombination, and Repair, Gene Expression, RNA Damage and Repair
12	**KATNA1**, KATNB1	1	Cellular Assembly and Organization, Cellular Function and Maintenance, Cellular Compromise
13	**NFX1**, SERTAD1	1	Cancer, Cellular Growth and Proliferation, Reproductive System Disease
14	**KCNN1**, KCNN2	1	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Molecular Transport
15	**IGF1R**, IVNS1ABP	1	Cancer, Cell Death, Cell-To-Cell Signaling and Interaction
16	PIN4, **WDR12**	1	Cell Signaling, Post-Translational Modification, Protein Folding
17	CEBPA, **EXTL2**	1	Carbohydrate Metabolism, Cell Cycle, Connective Tissue Development and Function
18	**TNFRSF19L**, TRAF1	1	Dermatological Diseases and Conditions, Organismal Injury and Abnormalities, Cellular Growth and Proliferation
19	**ARSA**, SUMF1	1	Genetic Disorder, Lipid Metabolism, Metabolic Disease
20	**RNF139**, VHL	1	Cancer, Cell Cycle, Cell Morphology
21	BCHE, **CLPS**, PNLIP	1	Respiratory Disease, Small Molecule Biochemistry, Lipid Metabolism
22	MPHOSPH6, NCBP1, **PARN**	1	RNA Post-Transcriptional Modification, Cellular Growth and Proliferation, Reproductive System Development and Function
23	KISS1, **KISS1R**, PLCB1	1	Cancer, Cellular Movement, Lipid Metabolism
24	PEX5, PEX10, **PEX12**, PEX19	1	Genetic Disorder, Metabolic Disease, Cellular Assembly and Organization
25	DRD4, KCNJ3, KCNJ5, KCNJ6, **KCNJ9**	1	Molecular Transport, Psychological Disorders, Behavior

Gene names in BOLD are the ones that are the differentially expressed genes.